Claims

1. A method of mixing heterologous genes in expression cassettes located on artificial chromosomes said method comprising the steps of
providing two initial populations of cells that can mate with each other,
said initial populations comprising at least 2 cells in each population, and at least two cells in each population having different combinations of heterologous genes and/or different combinations of expression cassettes,
each cell comprising at least a first type of artificial chromosome, the at least first type of artificial chromosome comprising both at least two expression cassettes comprising heterologous genes and at least one selectable marker,
the selectable markers being allocated to artificial chromosomes so that each type of artificial chromosome from each population can be individually selected for,
mating the cells with each other, and
selecting mated cells that carry at least a subset of the selectable markers present on the artificial chromosomes in the two initial populations.

2. The method of claim 1, further comprising causing the selected mated cells to undergo meiosis.

3. The method according to claim 2, where meiosis is performed under conditions where cells without artificial chromosomes and cells that have not undergone meiosis do not survive.

4. The method according to claim 1, wherein the subset of markers selected for comprises at least one marker from and artificial chromosome in each of the initial populations to ensure selection of mated cells.

5. The method according to any of the preceding claims, wherein the selection for a subset of the selectable markers includes selecting at least 70 % of all diploid types present in the mated population.

6. The method according to claim 5, wherein the selection of a subset of the selectable markers includes selecting at least 80 % of all diploid types present in the mated population.

7. The method according to any of the preceding claims, further comprising screening mated cells for one or more parameters related to a desired functionality(ies) and selecting cells having a predefined selection criterion(a) to undergo meiosis and mating.

8. The method according to any of the preceding claims, further comprising screening cells that have undergone meiosis for at least one parameter related to a desired functionality(ies) and selecting cells having a predefined selection criterion(a) to undergo mating and meiosis.

9. The method according to claims 7 or 8, wherein the selection threshold(s) associated with the desired functionalty(ies) is increased for each round of mating and meiosis.

10. The method according to any of the preceding claims, wherein one screening for more than one parameter is performed for at least one round of mating and meiosis.

11. The method according to any of the preceding claims, further comprising repeating the steps of claims 1 and 2 at least twice,.

12. The method according to any of the preceding claims, further comprising subjecting the populations of cells to physical isolation of artificial chromosomes from the populations for every 4-5 rounds of meiosis and selection, and transferring the isolated artificial chromosomes into new host cells.

13. The method according to claim 12, wherein physical isolation comprises amplification of artificial chromosomes in the host cells.

14. The method according to claim 12, wherein physical isolation comprises cutting expression cassettes from concatamers of expression cassettes on artificial

chromosomes and re-assembling expression cassettes into an artificial chromosome vector backbone and transforming these into new host cells.

15. The method according to any of the preceding claims, further comprising separating cells of the two mating types from each other after meiosis.

16. The method according to any of the preceding claims, further comprising mixing spores from different populations prior to mating.

17. The method according to any of the preceding claims, further comprising storing a sub-population of mated and selected cells, while another sub-population undergoes further meiosis and mating.

18. The method according to any of the preceding claims 2 to 17, further comprising adding a further population of cells with types of artificial chromosomes comprising at least two expression cassettes with heterologous genes, the cells being capable of mating with the cells that have undergone mating and meiosis, the further population comprising at least 2 cells with combinations of expression cassettes different from the combinations in the cells of the initial population, the artificial chromosomes of said further population carrying at least one selectable marker.

19. The method according to claim 18, wherein the types of artificial chromosomes of said further population have the same markers as the initial populations.

20. The method according to claim 18, wherein the further population comprises a 50/50 mixture of cells of the two mating types of the initial populations.

21. The method according to claim 18, wherein the further population comprises cells of one of the mating types of the initial populations.

22. The method according to any of the preceding claims 17-21, further comprising screening an earlier stored sub-population together with a population that has undergone at least one further round of meiosis and mating at a higher selection

threshold than the previous screening, selecting cells above the higher selection threshold, and mating the selected cells with each other.

23. The method according to any of the preceding claims, wherein at least one of the two initial populations of cells that can mate with each other further carry at least a second type of artificial chromosome with expression cassettes comprising heterologous genes, the first and second types of artificial chromosome carrying at least one selectable marker so that said first and second type of artificial chromosome can be individually selected for.

24. The method according to claim 23, wherein at least one of the two initial populations of cells that can mate with each other further carry at least a third type of artificial chromosome with expression cassettes comprising heterologous genes, the first, second, and third types of artificial chromosome carrying at least one selectable marker so that said first, second, and third type of artificial chromosome can be individually selected for.

25. The method according to claim 24, wherein at least one of the two initial populations of cells that can mate with each other further carry at least a fourth type of artificial chromosome with expression cassettes comprising heterologous genes, the first, second, third, and fourth type of artificial chromosome carrying at least one selectable marker so that said first, second, third, and fourth type of artificial chromosome can be individually selected for.

26. The method according to any of the preceding claims, wherein the two initial populations of cells that can mate with each other carry from 1 to 10 types of artificial chromosomes, each type of artificial chromosome of each population carrying at least one selectable marker so that each of the types of artificial chromosomes from each of the two populations can be individually selected for.

27. The method according to claim 18, wherein the further population of cells with artificial chromosomes capable of mating with the cells that have undergone mating and meiosis carry from 1 to 10 types of artificial chromosomes, each type of artificial chromosome of said further population carrying at least one

selectable marker so that each of the types of artificial chromosomes can be individually selected for.

28. The method according to any of the preceding claims, wherein each cell carries 2 artificial chromosomes per cell that can mate.

29. The method according to any of the preceding claims, wherein each cell carries 3 artificial chromosomes per cell that can mate.

30. The method according to any of the preceding claims, wherein each artificial chromosome carries at least two selectable markers, the selectable markers being allocated to artificial chromosomes so that each type of artificial chromosome from each population can be individually selected for.

31. The method according to claim 30, wherein at least one marker is located on the long arm of the artificial chromosomes.

32. The method according to any of the preceding claims, wherein each artificial chromosome comprises a common selectable marker, said selectable marker preferably being an auxotrophic marker.

33. The method according to any of the preceding claims, wherein the markers are selected from drug resistance, colour, morphology, resistance against electromagnetic radiation, salt tolerance, oxidative stress resistance fluorochrome probes and auxotrophy markers, more preferably auxotrophy markers.

34. The method according to any of the preceding claims, wherein the markers are sequence tags that can be detected by fluorescent probes/stains.

35. The method according to any of the preceding claims, wherein the markers are selected from the group consisting of NPT^{II}, LEU 2, TRP 1, HIS 3, LYS 2, URA 3, ADE 2, Amyloglucosidase, β-lactamase, CUP 1, $G418^{R}$, TUN^{R}, KILk1, C230, SMR1, SFA, $Hygromycin^{R}$, $methotrexate^{R}$, $chloramphenicol^{R}$, $Diuron^{R}$, $Zeocin^{R}$, $Canavanine^{R}$, ARG 4, THR, Luciferase, GUS, GFP, LUX.

36. The method according to any of the preceding claims, wherein the two initial populations are of different mating types.

37. The method according to any of the preceding claims, wherein the two initial populations have approximately the same number of cells.

38. The method according to any of the preceding claims 1-36, wherein the number of cells in one population is higher than the number of cells in the other population.

39. The method according to any of the preceding claims, wherein type of artificial chromosomes with the same marker or combination of markers differ with respect to combinations of expression cassettes comprising heterologous genes.

40. The method according to any of the preceding claims, wherein the species of cells are eukaryotic.

41. The method according to any of the preceding claims, wherein the species of cells are prokaryotic.

42. The method according to claim 40, wherein the species of cells are fungal cells.

43. The method according to claim 42, wherein the fungal cells are selected from a spore forming species.

44. The method according to claim 42, wherein the fungal cells are yeast cells.

45. The method according to claim 42, wherein the yeast cells are selected from the group comprising comprising baker's yeast, Kluyveromyces marxianus, K. lactis, Candida utilis, Phaffia rhodozyma, Saccharomyces boulardii, Pichia pastoris, Hansenula polymorpha, Yarrowia lipolytica, Candida paraffinica, Schwanniomyces castellii, Pichia stipitis, Candida shehatae, Rhodotorula glutinis, Lipomyces lipofer, Cryptococcos curvatus, Candida spp. (e.g. C.

palmioleophila), Yarrowia lipolytica, Candida guilliermondii, Candida, Rhodotorula spp., Saccharomycopsis spp., Aureobasidium pullulans, Candida brumptii, Candida hydrocarbofumarica, Torulopsis, Candida tropicalis, Saccharomyces cerevisiae, Rhodotorula rubra, Candida flaveri, Eremothecium ashbyii, Pichia spp., Kluyveromyces, Hansenula, Kloeckera, Pichia, Pachysolen spp., Schizosaccharomyces pombe (fission yeast), or Torulopsis bombicola.

46. The method according to claim 40, wherein the species of cells are plant cells or algae cells.

47. The method according to claim 40, wherein the species of cells are animal cells.

48. The method according to claim 41, wherein the species of cells are bacterial cells such as Escherichia coli, Bacillus subtilis, Streptomyces lividans, Streptomyces coelicolor, Pseudomonas aeruginosa, Myxococus xanthus, and wherein mating is conjugation.

49. The method according to any of the preceding claims, wherein the mated cells are diploid.

50. The method according to any of the preceding claims, wherein the mated cells are tetraploid.

51. The method according to any of the preceding claims, wherein the mated cells are hexaploid.

52. The method according to any of the preceding claims, wherein the expression cassettes are located on a nucleotide concatemer comprising in the 5'→3' direction a cassette of nucleotide sequence of the general formula

$$[rs_2\text{-SP-PR-X-TR-SP-}rs_1]_n$$

wherein

rs_1 and rs_2 together denote a functional restriction site,

SP individually denotes a spacer of at least two nucleotide bases,
PR denotes a promoter, capable of functioning in a cell,
X denotes an expressible nucleotide sequence,
TR denotes a terminator, and
SP individually denotes a spacer of at least two nucleotide bases, and
$n \geq 2$, and
wherein at least a first cassette is different from a second cassette.

53. The method according to any of the preceding claims, comprising expressible nucleotide sequences from at least one expression state.

54. The method according to any of the preceding claims, comprising nucleotide sequences from at least two expression states.

55. The method according to claim 52, wherein the rs_1-rs_2 restriction site of at least two cassettes are recognised by the same restriction enzyme,.

56. The method according to claim 52, wherein the rs_1-rs_2 restriction site of essentially all cassettes are recognised by the same restriction enzyme,.

57. The method according to any of the preceding claims, wherein substantially all expression cassettes on one artificial chromosome are different.

58. The method according to claim 52, wherein at least one expression cassette comprises an intron between the promoter and the expressible nucleotide sequence,.

59. The method according to any of the preceding claims, wherein the different combinations of expression cassettes comprises different promoters, and/or different expressible nucleotide sequences, and/or different spacers and/or different terminators and/or different introns.

60. The method according to claim 52, wherein n is at least 10,.

61. The method according to any of the preceding claims, wherein the artificial chromosome is selected from the group comprising a Yeast Artificial Chromosome, a mega Yeast Artificial Chromosome, a Bacterial Artificial Chromosome, a mouse artificial chromosome, a Plant Artificial Chromosome, a Mammalian Artificial Chromosome, an Insect Artificial Chromosome, an Avian Artificial Chromosome, a Bacteriophage Artificial Chromosome, a Baculovirus Artificial Chromosome, or a Human Artificial Chromosome.

62. The method according to claim 53 or 54, wherein the different expression states represent at least two different tissues, such as at least two organs, such as at least two species, such as at least two genera.

63. The method according to claim 62, wherein the different species are from at least two different phylae, such as from at least two different classes, such as from at least two different divisions, more preferably from at least two different sub-kingdoms, such as from at least two different kingdoms.

64. The method according to claim 62, wherein one species is a eukaryot and another species is a prokaryot.

65. The method according to any of the preceding claims, wherein the different combinations of heterologous genes and/or different combinations of expression cassettes on artificial chromosomes are designed to minimise the level of repeat sequences occurring.

66. A method of mixing heterologous genes in expression cassettes located on artificial chromosomes said method comprising the steps of
providing two initial populations of protoplasts or cells that can be fused,
said initial populations comprising at least 2 cells in each population, and at least two cells in each population having different combinations of heterologous genes and/or different combinations of expression cassettes,
each cell comprising at least a first type of artificial chromosome, said at least first type of artificial chromosome comprising both at least two expression cassettes comprising heterologous genes and at least one selectable marker,

the selectable markers being allocated to artificial chromosomes so that each type of artificial chromosome from each population can be individually selected for,

performing protoplast fusion and regeneration of cell walls or performing fusion of cells, and

selecting fused cells that carry at least a subset of the selectable markers present on the artificial chromosomes in the two initial populations.

67. The method according to claim 66, further comprising repeating the steps of claim 66.

68. The method according to claim 66, wherein the species of cells are selected from fungi, algae, and plants.

69. The method according to claim 66, wherein the species of cells are selected from prokaryots.

70. The method according to claim 66, wherein the species of cells are selected from animal cells, including human cells.

71. The method according to claim 66, wherein the species of cells are selected from plant, preferably carrot, Arabidopsis thaliana, Nicotiana spp., Nicotiana tabacum, maize, wheat, rice, soybean, tomato, peanut, potato, sugar beets, sunflower, yam, rape seed, conifers, and petunia.

72. The method according to any of the preceding claims 66 to 71, further comprising screening cells that result from protoplast fusion for a desired functionalty(ies) and selecting cells having the desired functionalty(ies) above a defined threshold, isolating protoplasts from these cells and performing protoplast fusion and cell regeneration on the selected cells.

73. The method according to claims 72, wherein the selection threshold(s) associated with the desired functionalty(ies) is increased for each round of protoplast isolation and fusion.

74. The method according to any of the preceding claims 66 to 73, further comprising storing a sub-population of cells regenerated from fused protoplasts, while another sub-population undergoes protoplast isolation and fusion.

75. The method according to claim 74, further comprising screening an earlier stored sub-population together with a population that has undergone at least one further round of protoplast isolation and fusion at a higher selection threshold than the previous screening, selecting cells above the higher selection threshold, and performing protoplast fusion on the selected cells.

76. The method according to any of the preceding claims 66 to 75, wherein at least one of the two initial populations of protoplasts that can fuse with each other further carries at least a second type of artificial chromosome with expression cassettes comprising heterologous genes, the first and second type of artificial chromosome from each population carrying at least one selectable marker so that said first and second type of artificial chromosome can be individually selected for.

77. The method according to any of the preceding claims 66 to 76, wherein selection of a subset of the selectable markers includes selection for at least 70 % of all fused cell types present in the fused population.

78. The method according to claim 77, wherein selectin of a subset of the selectable markers includes selecting at least 80 % of all fused cell types present in the fused population, such as at least 90%, for example at least 95%, such as at least 99%, for example 100%.

79. The method according to any of the preceding claims 66 to 78, further comprising repeating the steps of claim 66 at least twice, such as 3 times, for example 4 times, such as 5 times, for example 6 times, such as 7 times, for example 8 times, such as 9 times, for example 10 times, such as 11 times, for example 12 times, such as 13 times, for example 14 times, such as 15 times, for example 16 times, such as 17 times, for example 18 times, such as 19 times, for example 20 times, such as 25 times, for example at least 30 times, such as at least 40 times, for example at least 50 times, such as at least 75 times, for

example at least 100 times, such as at least 200 times, for example at least 300 times, such as at least 500 times, for example at least 1000 times.

80. The method according to any of the preceding claims, further comprising subjecting the populations of cells to physical isolation of artificial chromosomes from the populations for every 2-3 rounds of meiosis and selection, and transferring the isolated artificial chromosomes into new host cells.

81. The method according to any of the preceding claims 66-80, wherein the two initial populations of cells carry from 1 to 10 types of artificial chromosomes, each type of artificial chromosome of each population carrying at least one selectable marker so that each of the types of artificial chromosomes from each of the two populations can be individually selected for.

82. The method according to any of the preceding claims 66 to 81, further comprising adding a further population of cells with artificial chromosomes comprising at least two expression cassettes with heterologous genes, the cells being capable of fusing with the cells that have undergone fusion, the further population comprising at least 2 cells with combinations of expression cassettes different from the combinations in the cells of the initial population, the artificial chromosomes of said further population carrying at least one selectable marker.

83. The method according to claim 82, wherein the further population of cells with artificial chromosomes capable of fusing with the cells that have undergone mating and meiosis carry from 1 to 10 types of artificial chromosomes, each type of artificial chromosome of said further population carrying at least one selectable marker so that each of the types of artificial chromosomes can be individually selected for.

84. The method according to any of the claims 66-83, wherein each cell carries 2 artificial chromosome per cell/protoplast to be fused.

85. The method according to any of the claims 66-83, wherein each cell carries 3 artificial chromosome per cell/protoplast to be fused.

86. The method according to any of the preceding claims 66-85, comprising the features of any of the claims 30-36, 37-39, and 52-65.

87. A method of mixing heterologous genes in expression cassettes located on artificial chromosomes, said method comprising the steps of

a) obtaining at least one population of cells, the cells of said at least one population comprising

a concatemer of expression cassettes of the following formula:

$[rs_2\text{-SP-PR-X-TR-SP-}rs_1]_n$

wherein

rs_1 and rs_2 together denote a restriction site,

SP individually denotes a spacer,

PR denotes a promoter, capable of functioning in the cells,

X denotes an expressible nucleotide sequence,

TR denotes a terminator, and

$n \geq 2$,

the cells differing from each other with respect to combinations of expressible nucleotide sequences and/or promoters,

b) isolating at least some of the cassettes of the selected cells by cutting the concatemers with a restriction enzyme cleaving rs_1rs_2,

c) amplifying at least some of the isolated cassettes,

d) assembling the expression cassettes of step c) into artificial chromosomes, and

e) optionally transferring the artificial chromosomes into host cells.

88. The method according to claim 87, wherein amplification of isolated cassettes comprises PCR with primers for tagging rs_1 and rs_2.

89. The method according to claim 87, wherein amplification of isolated cassettes comprises inserting isolated cassettes into a vector having a cloning site compatible with rs_1rs_2 and multiplying this vector in a suitable host.

90. The method according to claim 87, further comprising adding further cassettes for the assembly step.

91. The method according to any of the preceding claims 87 to 90, further comprising screening cells with assembled artificial chromosomes for a desired functionalty(ies) and selecting cells having the desired functionalty(ies).

92. The method according to claim 91, further comprising subjecting the selected cells to further isolation and amplification of cassettes and assembly of artificial chromosomes.

93. A method for mixing heterologous genes in expression cassettes located on artificial chromosomes, said method comprising the steps of

providing two initial populations of cells,
said initial populations comprising at least 2 cells in each population, and at least two cells in each population having different combinations of heterologous genes and/or different combinations of expression cassettes,
each cell comprising at least a first type of artificial chromosome, the at least first type of artificial chromosome comprising both at least two expression cassettes comprising heterologous genes and at least one selectable marker,
the selectable markers being allocated to artificial chromosomes so that each type of artificial chromosome from each population can be individually selected for,
mating the cells with each other,
amplifying the artificial chromosomes in the host cells,
isolating the artificial chromosomes;
mixing the isolated artificial chromosomes,
transferring subsets of said isolated and mixed artificial chromosomes into host cells, and
selecting cells that carry at least a subset of the selectable markers present on the artificial chromosomes in the two initial populations.

94. The method according to claim 93, further comprising repeating the mixing process at least once, more preferably at least twice, more preferably at least three times, such as at least four times, for example at least 5 times, such as at least 6 times, for example at least 7 times, such as at least 8 times, for example

at least 9 times, such as at least 10 times, for example at least 15 times, such as at least 20 times, for example at least 25 times.

95. The method according to claim 93, wherein the host cells into which the subsets of mixed type of artificial chromosomes are transferred already contain artificial chromosomes with expression cassettes with heterologous genes.

96. A method of mixing expressible nucleotide sequences, said method comprising the steps of

a) obtaining at least one population of cells, the cells of said at least one population comprising at least one expression cassettes of the following formula:

$[rs_2\text{-SP-PR-rs1'-X-rs2'-TR-SP-}rs_1]_n$

wherein

rs_1 and rs_2 together denote a restriction site,

rs1' and rs2' together denote a different restriction site,

SP individually denotes an optional spacer,

PR denotes a promoter, capable of functioning in the cells,

X denotes an expressible nucleotide sequence,

TR denotes a terminator, and

$n \geq 2$,

b) isolating at least some of the expressible nucleotide sequences of the selected cells by cutting the cassettes with a restriction enzyme cleaving rs1'rs2', or by amplifying the sequences with primer pairs templating sequences in rs1' and rs2',

c) re-inserting the expressible nucleotide sequences into other similar backbone,

d) re-mixing the expression cassettes, and

e) transferring the re-expression cassettes into host cells.

97. The method according to claim 96, wherein the isolated expressible nucleotide sequences are inserted into primary vectors comprising a nucleotide sequence cassette of the general formula in 5'→3' direction:

[RS1-RS2-SP-PR-CS-TR-SP-RS2'-RS1']

wherein

RS1 and RS1' denote restriction sites,
RS2 and RS2' denotes restriction sites different from RS1 and RS1',
SP individually denotes a spacer sequence of at least two nucleotides,
PR denotes a promoter,
CS denotes a cloning site,
TR denotes a terminator.

98. The method according to claim 96 or 97, further comprising mixing artificial chromosomes by the steps of
providing two initial populations of cells that can mate with each other,
said initial populations comprising at least 2 cells in each population, and at least two cells in each population having different combinations of expression cassettes as defined in claim 96,
each cell comprising at least a first type of artificial chromosome, the at least first type of artificial chromosome comprising both at least two expression cassettes comprising heterologous genes and at least one selectable marker,
the selectable markers being allocated to artificial chromosomes so that each type of artificial chromosome from each population can be individually selected for,
mating the cells with each other, and
selecting mated cells that carry at least a subset of the selectable markers present on the artificial chromosomes in the two initial populations.

99. A method of mixing heterologous genes in expression cassettes located on plasmids said method comprising the steps of
providing two initial populations of cells that can mate with each other,
said initial populations comprising at least 2 cells in each population, and at least two cells in each population having different combinations of heterologous genes and/or different combinations of expression cassettes,
each cell comprising at least a first plasmid, the at least first plasmid comprising both at least two expression cassettes comprising heterologous genes and at least one selectable marker,
the selectable markers being allocated to plasmids so that each type of plasmid from each population can be individually selected for,
mating the cells with each other, and

selecting mated cells that carry at least a subset of the selectable markers present on the plasmids in the two initial populations.

100. The method according to claim 99, wherein the expression cassettes are located on a nucleotide concatemer comprising in the 5'→3' direction a cassette of nucleotide sequence of the general formula

$[rs_2\text{-SP-PR-X-TR-SP-}rs_1]_n$

wherein

rs_1 and rs_2 together denote a functional restriction site,
SP individually denotes a spacer of at least two nucleotide bases,
PR denotes a promoter, capable of functioning in a cell,
X denotes an expressible nucleotide sequence,
TR denotes a terminator, and
SP individually denotes a spacer of at least two nucleotide bases, and
$n \geq 2$, and
wherein at least a first cassette is different from a second cassette.

101. A method for mixing of expression cassettes comprising heterologous genes located on artificial chromosomes comprising using any of the methods according to any of the preceding claims in any sequential order.